



# UNIVERSITÀ DEGLI STUDI DI TORINO

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**Short-chain chitin oligomers from arbuscular mycorrhizal fungi trigger nuclear Ca<sup>2+</sup> spiking in *Medicago truncatula* roots and their production is enhanced by strigolactone**

Andrea Genre, Mireille Chabaud, Coline Balzergue Virginie Puech-Pagès, Mara Novero, Thomas Rey, Joëlle Fournier, Soizic Rochange, Guillaume Bécard, Paola Bonfante and David G. Barker

*Keywords:*

arbuscular mycorrhiza (AM);chitin oligomers;common SYM pathway;fungal–plant signalling;germinated spore exudates; *Medicago truncatula* ;nuclear calcium spiking;strigolactone

## *Summary*

The primary objective of this study was to identify the molecular signals present in arbuscular mycorrhizal (AM) germinated spore exudates (GSEs) responsible for activating nuclear  $\text{Ca}^{2+}$  spiking in the *Medicago truncatula* root epidermis.

*Medicago truncatula* root organ cultures (ROCs) expressing a nuclear-localized cameleon reporter were used as a bioassay to detect AM-associated  $\text{Ca}^{2+}$  spiking responses and LC-MS to characterize targeted molecules in GSEs.

This approach has revealed that short-chain chitin oligomers (COs) can mimic AM GSE-elicited  $\text{Ca}^{2+}$  spiking, with maximum activity observed for CO4 and CO5. This spiking response is dependent on genes of the common SYM signalling pathway (DMI1/DMI2) but not on NFP, the putative *Sinorhizobium meliloti* Nod factor receptor. A major increase in the CO4/5 concentration in fungal exudates is observed when *Rhizophagus irregularis* spores are germinated in the presence of the synthetic strigolactone analogue GR24. By comparison with COs, both sulphated and nonsulphated Myc lipochito-oligosaccharides (LCOs) are less efficient elicitors of  $\text{Ca}^{2+}$  spiking in *M. truncatula* ROCs.

We propose that short-chain COs secreted by AM fungi are part of a molecular exchange with the host plant and that their perception in the epidermis leads to the activation of a SYM-dependent signalling pathway involved in the initial stages of fungal root colonization.

## *Introduction*

Arbuscular mycorrhizal (AM) associations are ancient plant symbioses playing a central role in most land ecosystems, both by improving soil nutrient uptake and by contributing to protection against fungal pathogens. It has been proposed that the establishment of the AM endosymbiosis requires a molecular dialogue between the plant and fungal partners. Strong evidence suggests that a key component of this dialogue is the perception of host-secreted strigolactones, which activate fungal development and, in particular, the intense hyphal ramification in the vicinity of the root which precedes colonization of the root tissue (Akiyama et al., 2005; Besserer et al., 2006). However, the obligate biotrophy of AM fungi and the inability to perform genetics on these microbes have greatly hampered the characterization of the reciprocal fungal signalling molecules. Most of our current knowledge on symbiotic signalling derives from parallel studies on the *Rhizobium*–legume symbiosis, where it has been shown that these nitrogen-fixing bacteria synthesize specific decorated lipochito-oligosaccharides (LCOs) termed Nod factors (Dénarié & Cullimore, 1993). In addition, plant genetics has led to the characterization of several genes essential for initiating both root endosymbioses (MtDMI1, MtDMI2 and MtDMI3 in the case of the model legume *Medicago truncatula*), thus defining a shared ‘common SYM’ signalling pathway (Parniske, 2008). This pathway also relies on calcium-mediated signalling, and in particular the triggering of nuclear-associated  $\text{Ca}^{2+}$  spiking (Oldroyd & Downie, 2006). Upstream of the common SYM pathway, perception of Nod factors requires the MtNFP gene, encoding a LysM Receptor-Like Kinase (Arrighi et al., 2006). However, although NFP plays some role in host responses to Myc LCOs (Maillet et al., 2011; Czaja et al., 2012), it is not essential for AM colonization, since *nfp* mutants have a wildtype (WT) AM pheno-type (Ben Amor et al., 2003).

Since the bacterial symbiosis evolved more recently than the AM association (60 vs 450 million yr ago), it is currently thought that rhizobia have exploited the ancient mycorrhizal signalling pathway by mimicking symbiotic fungal signals. Therefore, by analogy with bacterial Nod factors, it has been proposed that AM fungi release signal molecules (Myc factors) essential for recognition of the fungal partner (Albrecht et al., 1999; Catoira et al., 2000). This has led to the hypothesis that modern legumes possess two separate branches that feed into the common SYM pathway, one for each type of root symbiont. Thus, hypothetical AM Myc factors should be able to elicit SYM pathway-dependent early plant responses that are specific to mycorrhizal colonization and their activity is likely to be independent on the NFP Nod factor receptor. In addition, and in contrast to Nod factors, Myc factors should be active on a wide variety of plant hosts (including, of course, nonlegumes), as there is no evidence for strict host specificity in the AM association.

A number of studies have indicated the production of diffusible fungal signals by either AM hyphae, germinating spores or mycorrhizal roots. The responses to these fungal signals include changes in intracellular calcium concentration (Navazio et al., 2007; Chabaud et al., 2011) and gene transcriptional activation (Kosuta et al., 2003; Weidmann et al., 2004; Drissner et al., 2007; Kuhn et al., 2010; Mukherjee & Ané, 2011; Ortu et al., 2012). Not all of these responses are dependent on the common SYM pathway, suggesting the existence of alternative signalling pathways and possibly also a variety of fungal factors. Recently, LCOs have been identified at low concentrations in mycorrhizal root exudates and in germinating AM spore exudates using bioassays originally established for Nod factor characterization (Maillet et al.,

2011). These so-called Myc LCOs are active in stimulating lateral root development and overall levels of mycorrhization. Furthermore, genetic studies have shown that, as for Nod factors, enhanced lateral root development in response to Myc LCOs is SYM pathway-dependent. Finally, this biological response is totally or partially dependent upon the NFP gene depending on the Myc LCO concentration.

Since  $\text{Ca}^{2+}$  signalling in the form of sustained intracellular oscillations is a central feature of the common SYM pathway, in vivo fluorescent  $\text{Ca}^{2+}$  reporters known as cameleons have proved to be particularly useful tools for evaluating cellular responses to microbial symbiotic signals in the host epidermis (Miwa et al., 2006; Kosuta et al., 2008). Purified rhizobial Nod factors elicit characteristically regular  $\text{Ca}^{2+}$  spiking in both the nucleus and cytoplasm of *M. truncatula* root hairs (Ehrhardt et al., 1996; Sieberer et al., 2009), whereas germinated spore exudates (GSEs) of the AM fungus *Gigaspora margarita* trigger nonregular spiking in root epidermal atrichoblasts of the same legume as well as in the nonlegume *Daucus carota* (Chabaud et al., 2011). Importantly, AM GSEs are active on *M. truncatula* root organ cultures (ROCs), consistent with the fact that ROCs can be successfully colonized by AM fungi. Furthermore, this AM-elicited calcium signalling is dependent on the common SYM genes DMI1 and DMI2 and independent of NFP. By contrast, rhizobia are unable to nodulate ROCs, and *Sinorhizobium meliloti* Nod factors fail to induce  $\text{Ca}^{2+}$  spiking in the ROC epidermis (Chabaud et al., 2011).

In this article, we have made use of ROCs expressing the nuclear-localized NupYC2.1 cameleon as a Nod-independent bioassay to identify and characterize potential symbiotic signal molecules of AM fungal origin. We have discovered that the nonregular spiking observed in response to *G. margarita* GSEs (Chabaud et al., 2011; this study) is also observed in response to exudates of *G. rosea* and the distantly related glomeromycete *Rhizophagus irregularis*. By contrast, GSEs of the biotrophic pathogen *Colletotrichum trifolii* are unable to trigger spiking in the *M. truncatula* ROC epidermis. The ROC/cameleon bioassay, coupled to mass spectrometric analysis, have together revealed that short-chain chitin oligomers (COs) elicit an analogous nuclear spiking signature and furthermore are present in the AM GSEs. Importantly, this CO-activated  $\text{Ca}^{2+}$  signalling is SYM pathway-dependent and NFP-independent. The addition of the synthetic strigolactone GR24 results in a striking increase both in the CO concentrations in *R. irregularis* GSEs and in the  $\text{Ca}^{2+}$  spiking activity of these exudates. These findings lead to the proposal that short-chain chitin oligomers generated by AM fungi are novel signals perceived in epidermal tissues of host plants and capable of activating an AM-related signalling pathway.

## *Materials and Methods*

### *Plant materials*

*Medicago truncatula* Gaertn. genotype Jemalong A17 and the derived mutants *nfp-2* (Arrighi et al., 2006), *dmi1-1* and *dmi2-2* (Catoira et al., 2000; Wais et al., 2000) were used in this study. *M. truncatula* ROCs expressing the 35S:NupYC2.1 construct (Sieberer et al., 2009) were obtained previously (Chabaud et al., 2011) and propagated in vertical dishes to favour a regular fishbone-shaped root system (Chabaud et al., 2002). Wildtype A17 and mutant *nfp-2* composite plants were obtained following transformation with *Agrobacterium rhizogenes* according to Boisson-Dernier et al. (2001). Two weeks after transformation, composite plantlets were transferred to

vertical square dishes ( $12 \times 12 \text{ cm}^2$ ) containing M medium (Bécard & Fortin, 1988) without sucrose, and covered with Biofolie 25<sup>TM</sup> (Lumox, Sarstedt, France) as described in Genre et al. (2005), and with the lower part of the dish wrapped in black plastic to protect the root from light. Roots from composite plants were used following 11–15 d of culture on M medium without sucrose.

Nongravitropic ROCs from the horticultural *Daucus carota* var. *sativus* expressing the 35S:NupYC2.1 were obtained previously (Chabaud et al., 2011) according to Bécard & Fortin (1988) and were grown horizontally. The *Arabidopsis thaliana* ecotype Columbia was transformed by flower dipping with *A. tumefaciens* strain GV3101 carrying the 35S:NupYC2.1 construct according to Clough & Bent (1998). Transformed T1 and T2 progenies were selected on 50 mg l<sup>-1</sup> kanamycin. Seeds from a single T2 line with high-level fluorescence were surface-sterilized and grown on M medium without sucrose in vertical dishes, with the lower part wrapped in black plastic. Lateral roots from 9-d-old plantlets were used for the test with chitin oligomers.

#### *Fungal materials and preparation of germinated spore exudate*

The AM fungi used in this study were *Gigaspora margarita* (BEG 34, International Bank for the Glomeromycota, University of Kent, UK), *Gigaspora rosea* (DAOM 194757, National Mycological Herbarium, Ottawa, Canada) and *Rhizophagus irregularis* (previously called *Glomus intraradices* (DAOM 197198); Krüger et al., 2012). Germinated spore exudates (GSEs) from *G. margarita* and *G. rosea* were prepared as described in Chabaud et al. (2011). Batches of 100 surface-sterilized *G. margarita* or *G. rosea* spores were placed in 1 ml of sterile H<sub>2</sub>O and incubated in the dark for 7 d at 30°C (2% CO<sub>2</sub>) to induce germination (germination rate > 90%). The GSE was recovered by pipetting, concentrated 10-fold using a lyophilizer and then stored at -20°C. One hundred microlitre aliquots (equivalent to 100 spores) were used in the ROC Ca<sup>2+</sup> spiking bioassay. Chitinase treatment of the *G. margarita* GSE was performed using 1 mg ml<sup>-1</sup> chitinase from *Streptomyces griseus* (ref C6137; Sigma-Aldrich) in sterile H<sub>2</sub>O for 16 h at room temperature. This enzyme complex degrades chitin to its monomer subunits.

To prepare *R. irregularis* GSEs,  $125 \times 10^3$  sterile spores (Agronutrition, Labège, France) were placed in 40 ml sterile H<sub>2</sub>O and incubated for 7 d in the dark at 30°C (2% CO<sub>2</sub>). When GSEs were prepared in the presence of strigolactone, GR24 was added to a final concentration of either 10<sup>-6</sup> or 10<sup>-8</sup> M. After incubation, the spores were removed by filtration (0.45 µm nitrocellulose filters; Millipore) and a butanol extraction performed on the GSE. The aqueous phase was lyophilized and resuspended in 100 µl H<sub>2</sub>O. Mass spectrometric analysis was performed on a 10 µl aliquot (400-fold concentrated compared with the GSE – equivalent to  $12.5 \times 10^3$  spores), and the same quantity used in the ROC Ca<sup>2+</sup> spiking bioassay after diluting 10-fold in H<sub>2</sub>O (40-fold concentrated compared with the GSE).

*Colletotrichum trifolii* spores were prepared as described in Torregrosa et al. (2004). A total of 107 spores were diluted in 100 ml of sterile H<sub>2</sub>O. After 24 h incubation at 24°C, the germinated spores were pelleted by centrifugation at 5000 g for 15 min and the GSE was recovered for analysis. *C. trifolii* GSEs were lyophilized and resuspended in 400 µl H<sub>2</sub>O. Mass spectrometry analysis was performed directly using 10 µl of the GSE (250-fold concentrated – equivalent to  $2.5 \times 10^5$  spores) and the ROC/Ca<sup>2+</sup> spiking bioassay using 100 µl of the GSE concentrate.

### *Bioassays for Ca<sup>2+</sup> spiking using roots expressing the NupYC2.1 reporter*

Several cm-long young lateral root segments (second-order) from either ROCs or composite plants, or young third-order lateral roots grown under Biofolie membrane (to limit root hair development), were excised and placed in a microchamber and treated with 100 µl of the solution to be tested as described in Chabaud et al. (2011). In the case of *Arabidopsis*, the entire plantlet was placed on the slide, with the whole root system in the microchamber. Confocal FRET-based ratio imaging for detecting and plotting relative changes of nuclear Ca<sup>2+</sup> concentrations corresponding to yellow fluorescent protein (YFP) to cyan fluorescent protein (CFP) fluorescence intensity changes over time (Miyawaki et al., 1997) was performed according to Sieberer et al. (2009) using a Leica TCS SP2 AOBS confocal laser-scanning microscope equipped with a long-distance HCX Apo L NA 40X 0.80 water-immersion objective or a HCX PL APO 40X 0.85 dry objective (Leica Microsystems GmbH, Wetzlar, Germany). Fluorescence intensity for both the CFP and YFP moieties of NupYC2.1 were collected, setting the pinhole diameter at 4–5 Airy units, exciting the probe at 458 nm (80% Ar laser) and recording the emitted fluorescence at 470–500 and 530–570 nm, respectively. Transmitted-light images were acquired simultaneously to confirm cell identity. Images were scanned at a resolution of 512 × 512 pixels and collected every 5 s over a period of 30 min after treatment. Average YFP and CFP fluorescence intensities were calculated for each nucleus using Leica LCS or Image J software (NIH, Bethesda, MD, USA). Values were then exported to a Microsoft Excel spreadsheet in order to calculate the YFP/CFP ratio for each time frame. Ratio values were then plotted over time to obtain a graphical representation of FRET intensity, corresponding to relative Ca<sup>2+</sup> concentration variations in the nucleoplasm. As previously reported (Chabaud et al., 2011), nuclear spiking in atrichoblasts was never observed in control untreated roots or before the addition of GSEs or chitin-based elicitors. Ca<sup>2+</sup> peaks were then counted manually and frequency distribution histograms of peak numbers were prepared as shown in Fig. 3 and Supporting Information Fig. S2. The total numbers of epidermal cell nuclei and independent roots analysed for each test are presented in Table S1.

### *Biochemicals used for bioassays*

Short-chain chitin oligomers were purchased from either Yaizu Suisankagaku Industry Corporation (Tokyo, Japan) or Seikagaku Biobusiness Corporation (Tokyo, Japan), and in addition CO3 and CO4 were kindly provided by Klaus Tietjen (Bayer Crop Science, Monheim, Germany). CO8 was kindly provided by Dr Naoto Shibuya from Meiji University, Kawasaki, Japan. The structure and purity of the commercial chitin oligomers were confirmed by MRM analysis, using two MRM transitions for each CO (see the following section). These analyses revealed that each CO was uncontaminated by either longer- or shorter-length COs and that there were no traces of any LCOs. In addition, the COs were fully N-acetylated. Stock solutions of chitin oligomers were prepared in H<sub>2</sub>O at 10<sup>−3</sup> M and stored at −20°C. *S. meliloti* Nod factor solutions were freshly prepared from a concentrated 10<sup>−3</sup> M stock (ethanol : water (1 : 1 (v/v))). Both sulphated and nonsulphated Myc LCOs were kindly provided by Eric Samain, Hugues Driguez, Sébastien Fort (CNRS, Grenoble, France) and Fabienne Maillet, Jean Dénarié (LIPM, Castanet-Tolosan, France). Myc LCOs were freshly prepared as C16:1 : C18:0 (1 : 1) mixtures from concentrated 10<sup>−4</sup> M stocks (acetonitrile : water (1 : 1 (v/v))). In the case of nonsulphated Myc LCOs,

dilutions were performed in such a way as to ensure that the final acetonitrile concentration was at least 0.5%. Finally, biochemical analysis was used to confirm the concentrations of the less water-soluble molecules (CO8 and nonsulphated LCOs) used in the bioassays.

#### *Biochemical analyses of GSEs*

Chito-oligosaccharides were detected using a 4000 Q Trap mass spectrometer with a Turbo V ESI source in the positive mode, coupled to an Agilent 1100 series HPLC system (Agilent Technologies Inc., Santa Clara, CA, USA). High-performance liquid chromatography (HPLC) separation was performed using a hypercarb column (5  $\mu$ m, 2  $\times$  100 mm; Hypercarb, Thermo; Suginta et al., 2009). For HPLC analysis, 10  $\mu$ l of GSE solutions was directly injected. Solutions of acetic acid : water (1 : 103 (v/v); A) and acetic acid : acetonitrile (1 : 103 (v/v); B) were pumped at 0.3 ml min<sup>-1</sup>. The gradient used was from 100% A to 100% B in 30 min.

The COs were identified in the MRM mode by monitoring the transitions from parent ion > common daughter ions (m/z: 204, 407, 610), and for quantification using the MRM transitions 628 > 204 m/z (CO3), 831 > 204 m/z (CO4), 1034 > 204 m/z (CO5), 1237 > 204 m/z (CO6), 1643 > 204 m/z (CO8). As an example, Fig. S1 illustrates a comparison of the MS/MS spectra for the CO4 standard and CO4 present in the AM fungal GSE. In addition, Table S2 compares the observed retention times and corresponding parent ions for short-chain CO standards and the GSE COs. Note that since retention times can vary slightly between experimental sessions, CO standards were always injected before and after the GSE samples. Blank controls confirmed the absence of residual signal carryover.

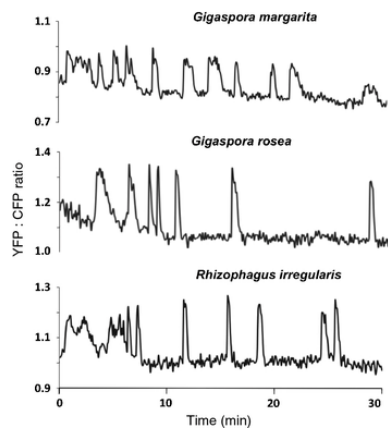
In order to determine the concentration of COs in the GSEs, a CO4 standard was added to the GSE before analysis. Since the CO4 signal obtained was threefold lower than expected due to the complexity of the matrix, this additional factor was integrated for determining the true concentrations of COs present within the GSEs.

#### *Results*

##### *Short-chain chitin oligomers mimic AM exudate-elicited Ca<sup>2+</sup> spiking activity in the host root epidermis*

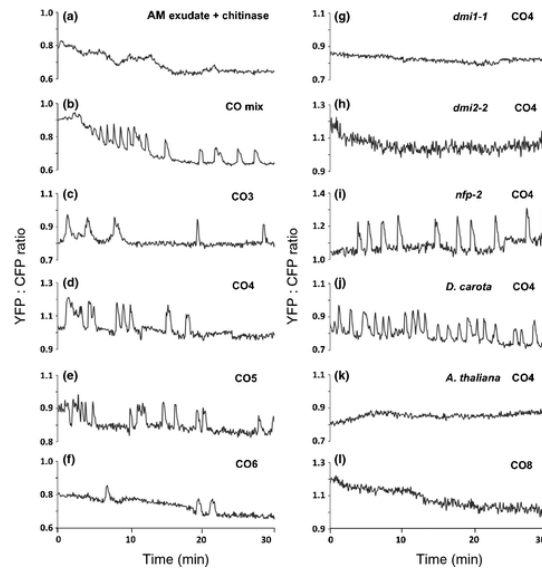
In order to determine to what extent the intracellular Ca<sup>2+</sup> signalling previously observed in the host root epidermis in response to *G. margarita* spore exudates (Chabaud et al., 2011) could be generalized to other AM fungal species, we isolated GSEs both from a second *Gigaspora* species (*G. rosea*) and from the distantly related *R. irregularis*. Fig. 1 shows that sustained Ca<sup>2+</sup> spiking could be observed in *M. truncatula* ROC atrichoblasts expressing the nuclear-localized cameleon NupYC2.1 when treated with either the *Gigaspora* or *Rhizophagus* GSEs. In all cases, the spiking profiles were highly irregular in terms of both the periodicity and the total number of spikes recorded over the 30 min period following the addition of the exudates. No correlation could be established between the AM species and the Ca<sup>2+</sup> spiking profile, which exhibited considerable cell-to-cell variation as previously reported for *G. margarita* (Chabaud et al., 2011).





**Figure 1.** Nuclear  $\text{Ca}^{2+}$  spiking in the *Medicago truncatula* epidermis in response to germinated spore exudates of various arbuscular mycorrhizal (AM) species. Nonregular  $\text{Ca}^{2+}$  spiking responses were observed in the nuclei of *M. truncatula* root organ culture (ROC) atrichoblasts expressing the nuclearameleon NupYC2.1 after the addition of germinated spore exudates (GSEs) from both *Rhizophagus* and *Gigaspora* AM species (see the 'Materials and Methods' section). Data are presented as yellow fluorescent protein : cyan fluorescent protein (YFP : CFP) ratios (arbitrary units) and roots were imaged every 5 s for 30 min following GSE treatment.

In order to characterize the bioactive molecules released by germinating AM fungal spores and responsible for triggering nonregular  $\text{Ca}^{2+}$  spiking in the host epidermis, we initially evaluated the chitinase-sensitivity of the *G. margarita* exudate. Fig. 2(a) shows that chitinase treatment completely abolished the spiking response, thus indicating a chitin-based structure for the putative symbiotic fungal factors. As part of a screen for potential candidate signals, initial tests performed on a mixture of chitin oligomers (COs) comprising 1–6 N-acetyl glucosamine residues revealed that the CO mix (10–8 M) was sufficient to activate sustained nonregular nuclear  $\text{Ca}^{2+}$  spiking in ROC epidermal cells (Fig. 2b). Subsequent experiments performed with the identical concentration of individual chitin oligomers (CO3–6; Fig. 2c–f) showed that maximal  $\text{Ca}^{2+}$  spiking was obtained with chitotetraose and chitopentaose (see Fig. S2a for a semiquantitative analysis). In all cases, the  $\text{Ca}^{2+}$  spiking profiles closely resembled the nonregular fungal GSE-elicited spiking. Finally, although all the  $\text{Ca}^{2+}$  responses presented in Fig. 2(a–f) were measured in ROC atrichoblast cells, short-chain COs also triggered nonregular spiking in root hair nuclei, as previously shown for *G. margarita* GSEs (Chabaud et al., 2011).



**Figure 2.** Short-chain chitin oligomers mimic the  $\text{Ca}^{2+}$  spiking activity of arbuscular mycorrhizal (AM) germinated spore exudates. *Medicago truncatula* root organ cultures (ROCs) expressing the NupYC2.1 cameleon were used as a bioassay to identify symbiotic AM signals. (a) Chitinase treatment of the *Gigaspora margarita* germinated spore exudate (GSE) suppresses  $\text{Ca}^{2+}$  spiking. (b) Nonregular  $\text{Ca}^{2+}$  spiking elicited in response to a 10–8 M mixture of chitin oligomers (CO1–6). (c–f) Representative  $\text{Ca}^{2+}$  spiking in response to individual short-chain COs (CO3–6) added at 10–8 M. (g–i) The CO-induced  $\text{Ca}^{2+}$  spiking response is dependent on the common SYM pathway genes DMI1 and DMI2, but independent of the putative Nod factor receptor NFP. (j, k) 10–8 M CO4 elicits spiking in the root epidermis of the nonlegume AM host *Daucus carota* but not the nonmycorrhizal plant *Arabidopsis thaliana*. (l) As shown in Supporting Information Fig. S1(b), >50% of atrichoblasts fail to respond to the defense response elicitor CO8 (10–8 M). Data are presented as yellow fluorescent protein : cyan fluorescent protein (YFP : CFP) ratios (arbitrary units).

Having established that short-chain COs were able to elicit nonregular  $\text{Ca}^{2+}$  spiking in *M. truncatula* ROCs, we then examined whether this response requires activation of the common SYM pathway. The fact that spiking failed to be elicited in either *dmi1* or *dmi2* mutant lines after addition of 10–8 M CO4 (Fig. 2g,h) implies that the common SYM pathway is indeed required for signal transduction upstream of nuclear  $\text{Ca}^{2+}$  signalling. Furthermore, our experiments also revealed that CO-elicited spiking was unaltered in an *nfp* mutant background (Fig. 2i). Thus, as for the *G. margarita* GSE, CO perception in ROCs is not dependent on the putative Nod factor receptor NFP.

In order to evaluate  $\text{Ca}^{2+}$  spiking responses to short-chain chitin oligomers in nonlegumes, we made use of available *D. carota* ROC lines expressing Nup-YC2.1 (Chabaud et al., 2011). We had previously observed that *G. margarita* GSEs were able to activate spiking in carrot roots (Chabaud et al., 2011), and Fig. 2(j) shows that 10–8 M CO4 is also an efficient trigger of  $\text{Ca}^{2+}$  spiking in this AM host. We then introduced the same nuclear-localized cameleon into the nonAM host *A. thaliana* (see the 'Materials and Methods' section). Significantly, chitotetraose failed to elicit spiking in the root epidermis of *A. thaliana* plants (Fig. 2k).

Finally, in order to compare  $\text{Ca}^{2+}$  spiking responses between short-chain COs and longer COs known to elicit plant defence responses (Shibuya & Minami, 2001; Shimizu et al., 2010), we treated *M. truncatula* ROCs with 10–8 M chitooctaose. Figs 2(l) and S2 show that CO8 is a very poor elicitor of spiking in *Medicago* roots, with responses even lower than those observed with CO6. In conclusion, short-chain chitin

oligomers (CO4/5 are the most active) are able to trigger nonregular Ca<sup>2+</sup> spiking in ROCs of AM host plants via the common SYM pathway and independently of NFP.

#### *Strigolactone boosts short-chain CO concentration in AM germinated spore exudates*

Having shown that short-chain COs elicit similar Ca<sup>2+</sup> spiking responses in epidermal tissues of AM hosts as compared with AM fungal GSEs, we then investigated the chitin oligomer composition of these exudates (see the 'Materials and Methods' section). Typical profiles obtained by multiple reaction monitoring (MRM) for CO<sub>3</sub>, CO<sub>4</sub>, CO<sub>5</sub> and CO<sub>6</sub> species are shown in Fig. S3. Quantitative data corresponding to the peak areas for each chitin oligomer are presented in Table 1, and reveal broadly similar concentrations for the four chitin oligomers. When these values are converted to concentrations using CO standards (see the 'Materials and Methods' section), our data reveal that CO<sub>3</sub>–6 are each present in the *R. irregularis* GSE in the concentration range  $0.3\text{--}1.2 \times 10^{-9}$  M. Since the Ca<sup>2+</sup> spiking experiments were performed with 40× concentrated GSEs, this means that the most active short-chain COs are present at a total concentration of c.  $5 \times 10^{-8}$  M. Similar results were obtained for GSEs of *G. rosea* (e.g.  $0.9 \times 10^{-9}$  M for CO<sub>4</sub>), although detailed analyses were more difficult to perform for *Gigaspora* species because of the limited availability of spores. Thus, the amounts of short-chain COs present in the various AM GSEs used in the Ca<sup>2+</sup> spiking bioassay are consistent with the spiking profiles obtained with purified  $10^{-8}$  M COs (Fig. 2).

**Table 1.** Concentrations of short-chain chitin oligomers (COs) present in *Rhizophagus irregularis* germinated spore exudates (GSEs) are significantly increased in the presence of the synthetic strigolactone GR24

	GSE – GR24 (counts × 10 <sup>-3</sup> )	GSE + GR24 (counts × 10 <sup>-3</sup> )	GR24 stimulation
CO <sub>3</sub>	33.5 ± 1.5	154 ± 7.0	× 4.6
CO <sub>4</sub>	44.7 ± 2.3	1690 ± 95	× 38
CO <sub>5</sub>	28.7 ± 1.7	1970 ± 120	× 69
CO <sub>6</sub>	17.0 ± 5.6	263 ± 18	× 15

The addition of GR24 ( $10^{-6}$  M) to the germination medium of *R. irregularis* spores leads to major increases in CO concentrations and, in particular, in the concentrations of CO<sub>4</sub>/5. The figures (total counts × 10<sup>-3</sup>) refer to the sum of the peak areas corresponding to each CO species (both α and β forms). Note that the CO quantification presented in this table was performed on a different *R. irregularis* GSE from that illustrated in Supporting Information Fig. S3.

n the case of the endosymbiotic rhizobial/legume association, the perception of secreted plant flavonoids by the soil bacterium is essential for optimizing Nod factor synthesis before root infection. To date, strigolactones are the only signalling components of host origin found to be essential for the AM symbiosis (Gomez-Roldan et al., 2008; Kretschmar et al., 2012). We therefore examined whether the addition of the synthetic strigolactone GR24 modified the concentrations of secreted COs present in the AM GSEs. The inclusion of  $10^{-6}$  M GR24 in the germination medium of *R. irregularis* spores (see the 'Materials and Methods' section) led to a major increase (c. 50-fold) in the concentrations of both chitotetraose and chitopentaose (Table 1). On the other hand, the increase was less important (five- to 15-fold) for the CO<sub>3</sub> and CO<sub>6</sub> species. In terms of nuclear Ca<sup>2+</sup> spiking activity, the positive effect of strigolactone can be seen by comparing the responses observed in atrichoblasts treated with GSEs prepared in either the absence or presence of  $10^{-6}$  M GR24 (Fig. S4). The major stimulation in short-chain chitin oligomer levels correlates with significantly enhanced spiking over the 30 min observation period. Indeed, when

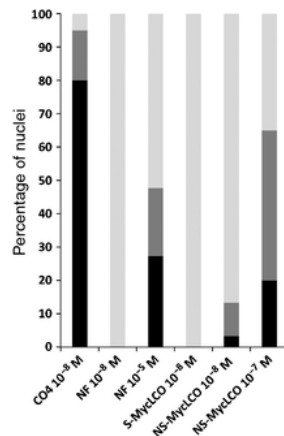
we add increasing concentrations of CO<sub>4</sub> (up to 10<sup>-6</sup> M) to *M. truncatula* ROCs, we observe an increase in the percentage of spiking atrichoblasts and also an overall increase in spiking intensity (not shown). In addition, it should be underlined that CO<sub>3-6</sub> concentrations in the *R. irregularis* GSE were still enhanced even when the GR24 concentration was lowered to 10<sup>-8</sup> M, but in this case increases were limited to between three and eightfold (not shown). By contrast, when 10<sup>-6</sup> M GR24 was included in the germination medium of *G. rosea* spores, we observed only modest increases (three- to fourfold) in short-chain CO concentrations. Finally, we were unfortunately unable to detect any LCOs in the butanol fractions of the *R. irregularis* GSEs tested. We presume that this is due to the low concentrations of Myc LCOs present in these GSEs (Maillet et al., 2011) and the fact that the MS sensitivity for the detection of LCOs is c. 100-fold lower than that for COs.

*Germinated spore exudates from a pathogenic fungus do not elicit Ca<sup>2+</sup> spiking in ROCs*

If, as these data suggest, the short-chain COs present in AM GSEs are primarily responsible for epidermal Ca<sup>2+</sup> spiking responses in *M. truncatula* roots, exudates from other chitin-rich biotrophic fungi would also be expected to elicit spiking in the ROC bioassay. To evaluate this, we prepared exudates from germinated spores of the *M. truncatula* hemibiotrophic pathogenic fungus *Colletotrichum trifolii* (see the 'Materials and Methods' section). Mass spectrometric analysis revealed that CO<sub>4</sub> was indeed present at similar concentrations in these exudates compared with AM GSEs (not shown). However, to our surprise, *C. trifolii* exudates failed to elicit any Ca<sup>2+</sup> spiking in the *M. truncatula* root epidermis and furthermore a negative response was obtained even when the exudate was supplemented with 10<sup>-8</sup> M purified CO<sub>4</sub> (Fig. S5). We therefore conclude that components of the pathogenic fungal exudate must be inhibiting the CO-elicited epidermal spiking.

*Short-chain COs are more active than LCOs in eliciting NFP-independent Ca<sup>2+</sup> spiking*

Having shown that short-chain COs are efficient elicitors of NFP-independent Ca<sup>2+</sup> spiking in *M. truncatula* ROCs, it was important to examine the activities of other known chitin-based symbiotic signals. We had previously demonstrated that 10<sup>-8</sup> M *S. meliloti* Nod factors were unable to trigger spiking in ROCs (Chabaud et al., 2011), and this result is presented in histogram format in Fig. 3. Indeed, even at the much higher concentration of 10<sup>-5</sup> M, these sulphated LCOs elicited only a limited spiking response (Fig. 3). Strikingly, this Nod factor-elicited spiking in the ROC epidermis resembles the nonregular Ca<sup>2+</sup> spiking triggered by AM GSEs and COs, and furthermore this response is NFP-independent (Fig. S6a,b). Together, this suggests that the identical signalling pathway is being activated by both 10<sup>-8</sup> M COs and the 103-fold higher concentrations of sulphated LCO.

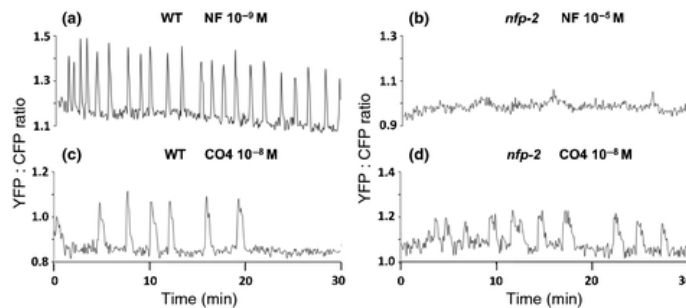


**Figure 3.** Ca<sup>2+</sup> spiking activities of various lipochito-oligosaccharides in the *Medicago truncatula* root organ culture epidermis. *Medicago truncatula* roots expressing NupYC2.1 were treated with different lipochito-oligosaccharides (LCOs) including *Sinorhizobium meliloti* Nod factors (NF) and both sulphated (S) and nonsulphated (NS) Myc LCOs at the indicated concentrations. For each treatment, between 15 and 45 atrichoblasts from at least two independent roots were imaged over 30 min (see also Supporting Information Table S1). The histograms show the percentage of nuclei with zero peaks (light grey), one to two peaks (dark grey) or  $\geq$  three peaks (black) throughout the entire 30 min imaging period.

Recently, Maillet et al. (2011) have described several LCO species present in low concentrations in exudates of AM-colonized carrot roots and in GSEs of *R. irregularis*. These LCOs are N-acylated with either C16:0 or C18:1 lipid chains and either sulphated or nonsulphated on the reducing end of the chitin backbone, but, unlike Nod factors, they are not O-acetylated (for a comparison of structures, see Fig. S7). Experiments using *M. truncatula* ROCs expressing the Nup-YC2.1 cameleon showed that 10<sup>-8</sup> M sulphated Myc LCOs (a mix of C16:0 and C18:1) were totally unable to activate Ca<sup>2+</sup> spiking (Fig. 3). However, as for the *S. meliloti* Nod factors, a low-level spiking response could be detected when the concentration of sulphated Myc LCOs was increased to 10<sup>-5</sup> M (not shown). By contrast, nonsulphated Myc LCOs elicited moderate spiking at 10<sup>-7</sup> M (responses in c. 50% of cells) and a low-level spiking could be detected at 10<sup>-8</sup> M (Fig. 3). Again, this nonregular spiking is maintained in an *nfp* mutant background (Fig. S6c,d), suggesting that Myc-LCOs are perceived in ROCs with a lower affinity by the same NFP-independent pathway as short-chain COs. In conclusion, these data indicate that the addition of the sulphate residue and the fatty acid chain to the chitin backbone leads to a significant reduction in NFP-independent Ca<sup>2+</sup> spiking activity in ROCs.

Although cameleon-expressing ROCs have proved to be excellent tools to identify and characterize AM-specific signalling molecules, because of the lack of interference with host responses to rhizobial LCOs, it was essential to examine Ca<sup>2+</sup> spiking responses elicited in whole plants by short-chain chitin oligomers. Since preliminary experiments had shown no differences in responses to either Nod factor or CO4 when applied to roots from either regenerated transgenic plants or WT composite plants (not shown), we present here a comparison of responses in both WT and *nfp* backgrounds exclusively using composite plants. A typical spiking profile elicited by 10<sup>-9</sup> M *S. meliloti* Nod factors in root hairs of composite *M. truncatula* plants expressing 35S:Nup-YC2.1 is shown in Fig. 4(a). As previously demonstrated, this characteristic spiking is totally absent in an *nfp* mutant background (Ben Amor et al., 2003), and we have confirmed that this is the case even when the Nod factor concentration is

increased to  $10^{-5}$  M (Fig. 4b). On the other hand, in line with the results obtained using ROCs,  $10^{-8}$  M CO4 elicits a less frequent and less regular  $\text{Ca}^{2+}$  spiking profile in root hairs of the whole WT plant (Fig. 4c). Furthermore, the fact that the CO4-induced spiking is maintained in an *nfp* background (Fig. 4d) confirms that the putative Nod factor receptor is not required for AM-related signal perception in whole plants. In conclusion, the characteristics of the  $\text{Ca}^{2+}$  spiking response elicited by short-chain chitin oligomers appear to be identical whether in *M. truncatula* plants or root organ cultures, whereas spiking responses to LCOs such as *S. meliloti* Nod factors differ radically in terms of spiking signature, required LCO concentration and NFP dependency. These results argue that COs and sulphated LCOs activate distinct  $\text{Ca}^{2+}$ -mediated signalling pathways in intact plants.



**Figure 4.** Comparison of  $\text{Ca}^{2+}$  spiking responses elicited by either Nod factors or chitotetraose on roots of both wildtype (WT) and *nfp* mutant plants. Composite *Medicago truncatula* plants expressing NupYC2.1 were used to compare the activities of Nod factors (NF) and chitin oligomers (COs) on whole plants. (a) Characteristic high-frequency regular spiking elicited in a root hair of a composite WT plant following treatment with  $10^{-9}$  M *Sinorhizobium meliloti* NF. (b) Absence of spiking in root hairs of a composite *nfp-2* mutant treated with  $10^{-5}$  M NF. (c) As is the case for root organ cultures (ROCs), nonregular and lower frequency spiking is induced in root hairs of WT composite plants treated with  $10^{-8}$  M CO4. (d) Similarly, and in contrast with NF,  $10^{-8}$  M CO4 elicits nonregular spiking in roots of *nfp-2* composite plants. Data are presented as yellow fluorescent protein : cyan fluorescent protein (YFP : CFP) ratios (arbitrary units) and roots were imaged every 5 s for 30 min following NF/CO4 treatment.

## Discussion

### Short-chain chitin oligomers activate an AM-related signalling pathway

Essential steps of host-microbe recognition for both the rhizobial and AM endosymbioses take place before root colonization when the appropriate microbial signals are perceived by root epidermal tissues. This leads to the activation of respective host signal transduction pathways, of which a conserved ‘common SYM’ segment includes both the triggering of intracellular  $\text{Ca}^{2+}$  spiking (Oldroyd & Downie, 2006) and its decoding via the essential nuclear-localized calcium and calmodulin-dependent kinase (CCaMK = DMI3). By making use of *M. truncatula* root organ cultures expressing a nuclear-localizedameleon as a Nod-independent bioassay for AM signals, we provide evidence in this article that short-chain chitin oligosaccharides are able to trigger an AM-related signalling pathway and that the two most active chitin oligomers (CO4 and CO5) are present in AM GSEs at concentrations compatible with the  $\text{Ca}^{2+}$  spiking activities of the exudates. Furthermore, major increases in both the CO4/5 concentrations and the associated  $\text{Ca}^{2+}$  spiking activities of *R. irregularis* exudates were observed when the synthetic strigolactone GR24 was included in the germination medium. Besserer et al. (2006, 2008) have shown that the mitochondrial and energy metabolism of AM fungi is

rapidly triggered in response to GR24. Therefore, the increase of COs observed here could simply be a consequence of a general metabolic boost. However, the fact that GR24-dependent increases in CO concentrations were significantly higher for the most active CO4 and CO5 compared with the other short-chain COs suggests that this qualitative modification in the exudates corresponds to a change in the physiological status of the AM fungus. By comparison, preliminary experiments failed to reveal any significant modification to short-chain CO concentrations in *C. trifolii* exudates in response to GR24 treatment (not shown). Genetic experiments have confirmed that the CO-elicited spiking in *M. truncatula* ROCs is dependent on the common SYM pathway genes MtDMI1 and MtDMI2, and furthermore that inactivation of the MtNFP gene, encoding the LysM RLK essential for Nod factor perception (Arrighi et al., 2006), did not modify the Ca<sup>2+</sup> spiking activity of short-chain chitin oligomers. This is consistent with the recent demonstration that the putative Nod factor receptors from *Lotus japonicus*, NFR1 and NFR5, can directly bind *Mesorhizobium loti* Nod factors in vitro with a significantly higher affinity compared to the chitin oligomer CO5 (Broghammer et al., 2012). Finally, the discovery that 10<sup>-8</sup> M CO4 is equally active in eliciting nuclear Ca<sup>2+</sup> spiking on roots of nonlegume hosts such as *D. carota* (and not on roots of the nonhost *A. thaliana*) provides additional evidence in favour of the hypothesis that chito-oligosaccharides are novel factors capable of activating an AM-related transduction pathway.

In this context, it is important to underline the fact that Walker et al. (2000) and Oldroyd et al. (2001) were the first to show that short-chain COs could elicit SYM pathway-dependent intracellular Ca<sup>2+</sup> spiking in root hairs of pea and *M. truncatula* seedlings respectively. At the time it was not envisaged that these molecules could be symbiotic fungal signals and the fact that experiments were performed on whole plants made it difficult to discriminate between 'Nod' and 'Myc' signalling. We show in this article that 10<sup>-8</sup> M CO4 can elicit nonregular and NFP-independent spiking in the root epidermis of whole *M. truncatula* plants as well as in ROCs. The fact that much higher CO4 concentrations (10<sup>-4</sup> M) were required to induce Ca<sup>2+</sup> spiking in the experiments performed by Oldroyd et al. (2001) on root hairs of 1-d-old seedlings suggests that the sensitivity to CO signals probably depends on various factors, including the stage of root development (Chabaud et al., 2011) and the epidermal cell type (trichoblasts vs root hairs).

How do plants distinguish between chitin-based pathogenic and symbiotic signals? Chitinaceous molecules are widespread microbial signals and their perception is essential for plants to discriminate between a variety of biotic stimuli. It is now known that receptors with extracellular LysM motifs are required for the recognition of both rhizobial LCOs in legumes (Limpens et al., 2003; Radutoiu et al., 2003) and chitin-based defence elicitors in model plants such as rice and *Arabidopsis* (Kaku et al., 2006; Miya et al., 2007). In the latter case, the length of the chitin oligomer appears to be crucial, since receptor affinity drops dramatically if the long-chain octamer is replaced by the short-chain tetramer or pentamer (Shibuya et al., 1996). By contrast, our experiments based on the *M. truncatula* ROC bioassay have revealed the reverse correlation, with maximal Ca<sup>2+</sup> spiking elicited by the chitin tetra/pentamers compared with the longer hexamer and octamer. Interestingly, Liu et al. (2012) have recently shown that activation of the AtCERK1 immune receptor requires long-chain chitin oligomer-induced dimerization, and that this activation is attenuated by the presence of short-chain oligomers such as CO5. Further work will, of course, be

necessary to attribute a role for short-chain CO signals in the AM endosymbiosis, unrelated to plant defence. Evidence for the role of LysM-type receptors during root mycorrhization has recently come from RNAi studies using the nonlegume *Parasponia* (Op den Camp et al., 2011), although it should be underlined that these findings relate to arbuscule formation in the root cortex rather than to the initial stages of root infection. Since the *M. truncatula* *nfp* mutant has a WT AM phenotype (Ben Amor et al., 2003), it is conceivable that alternative LysM-type receptor(s) are involved in perceiving short-chain COs in *M. truncatula*. Detailed phylogenetic analysis of this extensive gene family within *M. truncatula* (Arrighi et al., 2006) and across plant species (Zhang et al., 2007), coupled with inactivation approaches for candidate genes and the AM-specific ROC bioassay, should now provide the means of identifying potential Myc factor receptors in both legume and nonlegume mycorrhizal plants.

It is also of interest in this context that, despite the presence of short-chain COs in the GSE of the pathogenic hemibiotrophic fungus *C. trifolii*,  $\text{Ca}^{2+}$  spiking was not detected in *M. truncatula* roots following treatment with the *Colletotrichum* exudate. Since the addition of exogenous  $\text{CO}_4$  ( $10^{-8}$  M) was unable to compensate for this lack of response, we deduce that *C. trifolii* GSEs contain either  $\text{CO}_4$ -inhibitors/competitors, or other components that prevent  $\text{CO}_4$  activity. Finally, in the case of the hemibiotrophic association between the AM host *Oryza sativa* and *Magnaporthe oryzae*, Marcel et al. (2010) have shown that the rice CCaMK homologue to *M. truncatula* DMI3 is not essential for this pathogenic interaction. This therefore suggests that a SYM pathway-related oscillatory  $\text{Ca}^{2+}$  signal is not required to establish this pathogenic fungal–plant association. Together, these findings argue that both the perception of short chitin oligomers and the activation in the root epidermis of nuclear  $\text{Ca}^{2+}$  spiking via the SYM pathway are specifically required to set in motion the AM fungal symbiosis. Additional experiments to show the link between CO-induced  $\text{Ca}^{2+}$ -spiking and effective root colonization are required to confirm this hypothesis.

#### Roles for different chitin-based signals in the AM symbiosis

After many years of obscurity, the molecular language used by AM fungi to communicate to their plant hosts is finally emerging. Maillet et al. (2011) have identified several species of Myc LCOs present at low concentrations in exudates of AM-colonized carrot roots and *R. irregularis* GSEs. These Myc LCOs, as is the case for their rhizobial counterparts, are active in stimulating lateral root development (Maillet et al., 2011), and, as a consequence, capable of increasing the root surface available for mycorrhizal colonization. Depending upon the concentration of Myc LCOs, this biological activity is partially or totally dependent on the putative Nod factor receptor NFP (Maillet et al., 2011).

We now show that additional chitinaceous signals of AM origin in the form of nondecorated short-length chitin oligomers are able to activate a  $\text{Ca}^{2+}$ -mediated signalling pathway in root epidermal tissues, most probably via a distinct LysM-type receptor. Our experiments using *M. truncatula* ROCs demonstrate that short-chain COs are more active than either sulphated or nonsulphated Myc LCOs in triggering AM-dependent  $\text{Ca}^{2+}$  spiking. Conversely, it has been shown that  $10^{-8}$  M chitin tetraose is unable to stimulate root branching in *M. truncatula* plants (Oláh et al., 2005) and that sulphated LCOs are more active than nonsulphated LCOs in



stimulating lateral root development (Maillet et al., 2011). In this context, a recent article describes a transcriptomic analysis of host gene expression in *M. truncatula* roots in response to either Nod factors or Myc LCOs (Czaja et al., 2012). Despite the overall low levels of gene induction, the authors demonstrate that Myc LCO-induced gene expression is dependent on the NFP gene. This finding further underlines differences in the mechanism of host perception for AM-generated COs and LCOs. Together, this suggests that AM fungi may use two different chitin-based, signals as compared with a single multifunctional LCO for rhizobial species. Finally, it is striking that irregular  $\text{Ca}^{2+}$  spiking in the *Medicago* epidermis is elicited by AM fungal contact at sites of hyphopodia formation (Chabaud et al., 2011), by all the *Gigaspora* and *Rhizophagus* GSEs tested so far (Fig. 1) and also by purified short-chain COs (Fig. 2). It remains to be determined what this irregular spiking signifies, and whether it contributes to the specificity of downstream host cellular responses.

After spore germination in the soil, AM hyphae undergo intensive ramification in the immediate vicinity of host roots, probably due to the perception of secreted strigolactones. This ramification amplifies direct hyphal contact with the root outer surface and the differentiation of the preinfection adhesion structures known as hyphopodia. Mutations in the common SYM signalling pathway of *M. truncatula* result in failure to elaborate the intracellular prepenetration apparatus (PPA) following hyphopodium formation (Genre et al., 2005) and this presumably leads to infection arrest at the root surface. It is therefore likely that critical fungal–plant signalling takes place at this particular stage of the interaction, and this is supported by the observation of intense  $\text{Ca}^{2+}$  spiking within nuclei of atrichoblasts directly contacted by hyphopodia (Chabaud et al., 2011). The findings reported in this paper suggest that simple chitin oligomers (or closely related molecules) may be generated at the fungal–plant interface as part of the two-way preinfection molecular exchange involving strigolactones. From an evolutionary perspective, it is conceivable that the establishment of the dialogue between AM fungi and primitive host plants may have exploited simple products of their chitin metabolism as unsophisticated signals. This is also consistent with the fact that the AM association is both widespread and with no apparent host–fungal specificity. In addition to the triggering of common SYM pathway-dependent nuclear  $\text{Ca}^{2+}$  spiking, it will, of course, be important in the future to identify downstream mycorrhization-specific genes activated by short-chain COs. It is also an open question as to whether other important signalling molecules are generated at the fungal–host interface created after hyphopodium contact and adhesion. For example, the presence of surface signals, the secretion of additional effectors, and localized increase in exudate concentration could all contribute to the successful recognition of AM fungi as endosymbionts and their subsequent host-regulated entry into the plant root.

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